

University of Groningen

De Novo Peroxisome Biogenesis in *Penicillium Chrysogenum* Is Not Dependent on the Pex11 Family Members or Pex16

Opalinski, Lukasz; Bartoszewska, Magdalena; Fekken, Susan; Liu, Haiyin; de Boer, Rinse; van der Klei, Ida; Veenhuis, Marten; Kiel, Jan A. K. W.

Published in:
 PLoS ONE

DOI:
 [10.1371/journal.pone.0035490](https://doi.org/10.1371/journal.pone.0035490)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 2012

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Opalinski, L., Bartoszewska, M., Fekken, S., Liu, H., de Boer, R., van der Klei, I., Veenhuis, M., & Kiel, J. A. K. W. (2012). De Novo Peroxisome Biogenesis in *Penicillium Chrysogenum* Is Not Dependent on the Pex11 Family Members or Pex16. *PLoS ONE*, 7(4), [e35490]. <https://doi.org/10.1371/journal.pone.0035490>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Supplementary Materials and Methods

Penicillium chrysogenum growth conditions

For transformation, *P. chrysogenum* hyphae were cultivated in YGG medium containing 0.8 % KCl, 1.6 % glucose, 0.67 % yeast nitrogen base (Difco), 0.15 % citric acid, 0.6 % K₂HPO₄, 0.2 % yeast extract, pH 6.2, with addition of 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

For biochemical and ultrastructural studies, *P. chrysogenum* cells were cultivated in penicillin production medium (PPM; [1]) containing 7.5 % lactose, 0.5 % ammoniumacetate, 0.4 % Na₂SO₄, 0.4 % urea, 50 mM potassiumphosphate buffer, 0.05 % phenylacetic acid (or phenoxyacetic acid), and 4 ml/L of a trace element solution, pH 6.3. The trace-element solution contained per liter: 34.6 g EDTA.2H₂O, 43.76 g Na₃citrate.2H₂O, 24.84 g FeSO₄.7H₂O, 256.4 g MgSO₄.7H₂O, 12.4 mg H₃BO₃, 12.4 mg Na₂MoO₄.2H₂O, 0.64 g CuSO₄.5H₂O, 2.52 g ZnSO₄.7H₂O, 0.64 g CoSO₄.7H₂O, 3.04 g MnSO₄.H₂O and 1.24 g CaCl₂, pH 6.5.

For peroxisome proliferation, *P. chrysogenum* cells were cultivated in PPM supplemented with 0.1 % oleic acid and 0.05 % Tween 80.

P. chrysogenum ble^R transformants were isolated on phleomycin selection plates containing: 1 % yeast nitrogen base, 0.225 % citric acid, 0.9 % K₂HPO₄, 0.1 % yeast extract, 2 % glucose and 2 % agar, and 1 ml/L of the trace element solution, pH 7, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml phleomycin (Invivogen). For protoplast regeneration, 28.7 % sucrose was added.

P. chrysogenum AmdS⁺ transformants were isolated on plates with acetamide as sole source of nitrogen [2], which contained 0.3 % NaCl, 0.05 % MgSO₄.7H₂O, 0.001 % FeSO₄.7H₂O, 1 % glucose, 10 mM potassiumphosphate buffer pH 6.8, 0.1 % acetamide, 15 mM CsCl₂ and 2 % agar, supplemented with 34.2 % sucrose for regeneration of protoplasts.

P. chrysogenum mycelia were allowed to sporulate on R-agar plates containing 0.52 % v/v glycerol, 0.75 % v/v beet molasses, 0.5 % yeast extract, 300 mM NaCl, 0.2 mM MgSO₄.7H₂O, 0.44 mM KH₂PO₄, 3.3 µM NH₄Fe(SO₄)₂.12 H₂O, 0.4 µM CuSO₄.5H₂O, 1.45 mM CaSO₄.2H₂O and 2 % agar.

To select on removal of the *amdS* gene (by replication slippage [3]), *P. chrysogenum* spores were plated on fluoracetamide selection agar containing 0.3 % NaCl, 0.05 % MgSO₄.7H₂O, 0.001 %

FeSO₄·7H₂O, 1 % glucose, 10 mM potassiumphosphate buffer pH 6.8, 0.1 % fluoroacetamide, 0.03 % urea and 2 % agar.

The growth of *P. chrysogenum* mutants on oleic acid plates was analyzed using mineral medium [4] without yeast extract, supplemented with 0.1 % oleic acid and 0.05 % Tween 80. As a control mineral medium supplemented with 0.5 % glucose was used.

Construction of plasmids and strains

Strains, plasmids and oligonucleotides are listed in **Tables S1, S2 and S3**, respectively.

1. *P. chrysogenum* deletion strains

Construction of a *P. chrysogenum* pex11 deletion strain (Δ pex11 GFP.SKL)

Two plasmids allowing deletion of *P. chrysogenum* *pex11* were constructed using Multisite Gateway technology (Invitrogen). First, the 5' and 3' flanking regions of the *pex11* gene were amplified by PCR with primer combinations BB-JK005+BB-JK006 and BB-JK051+BB-JK052, respectively, using *P. chrysogenum* DS17690 genomic DNA as template. The resulting 1.2 kb (5') and 2.1 kb (3') fragments were then recombined into the Gateway vectors pDONRTM P4-P1R and pDONRTM P2R-P3, resulting in plasmids pBBK-005 and pBBK-014, respectively. Both plasmids were recombined together with either plasmid pENTR221-*amdS* or plasmid pENTR221-*niaD*_{F1}-*amdS*-*niaD*_{F2} and pDESTTM R4-R3, resulting in formation of the final deletion constructs pBBK-015 and pBBK-017, respectively. For the single *pex11* deletion strain, plasmid pBBK-015 was linearized with *Aat*II and transformed into *P. chrysogenum* *hdfA* GFP.SKL protoplasts. *AmdS*⁺ transformants were selected and analysed by colony PCR using primers BB-JK053 and P_{gpdA}-rev. These should only amplify a fragment of 1.3 kb upon correct deletion of *pex11*. Finally, Southern blotting was used to confirm correct deletion. Multiple independent deletion strains were obtained and used for further study.

Construction of a *P. chrysogenum* pex11B deletion strain (Δ pex11B GFP.SKL)

Two plasmids allowing deletion of *P. chrysogenum* *pex11B* were constructed using Multisite Gateway technology as follows: First, the 5' and 3' flanking regions of *pex11B* were amplified by PCR with primer combinations BB-JK021+BB-JK022 and BB-JK023+BB-JK024,

respectively, using DS17690 genomic DNA as template. The resulting 2.5 kb (5') and 2.5 kb (3') fragments were then recombined into the Gateway vectors pDONRTM P4-P1R and pDONRTM P2R-P3, respectively, resulting in plasmids pDONR 4-1 (PcPEX11B) and pDONR 2-3 (PcPEX11B), respectively. Both plasmids were recombined together with plasmids pENTR221-phleo or pENTR221-niaD_{F1}-amdS-niaD_{F2} and pDESTTM R4-R3, resulting in formation of the deletion constructs pSAL1.1 and pBBK-020, respectively. For the single *pex11B* deletion strain, plasmid pSAL1.1 was linearized with *NotI* and transformed into *P. chrysogenum hdfA* GFP.SKL protoplasts. Phleomycin resistant transformants were selected and analysed by colony PCR using primers BB-JK025 and phleo-rev. These should only amplify a fragment of 3.3 kb upon correct deletion of *pex11B*. Finally, Southern blotting was used to confirm the correct deletion. Multiple independent deletion strains were obtained and used for further study.

Construction of a P. chrysogenum pex11C deletion strain (Δ pex11C GFP.SKL)

A plasmid allowing deletion of *P. chrysogenum pex11C* was constructed using Multisite Gateway technology as follows: First, the 5' and 3' flanking regions of *pex11C* were amplified by PCR with primer combinations attb4-Ppex11c.fw+attb1-Ppex11c.rev and attb2-Tpex11c-fw+attb3-Tpex11c.rev, respectively, using DS17690 genomic DNA as template. The resulting 1.25 kb (5') and 1.25 kb (3') fragments were then recombined into the Gateway vectors pDONRTM P4-P1R and pDONRTM P2R-P3, respectively, resulting in plasmids pSUS26 and pSUS27, respectively. Both plasmids were recombined together with plasmids pENTR221-phleo and pDESTTM R4-R3, resulting in formation of the deletion construct pSUS28. For the *pex11C* deletion strain, a 5.5 kb fragment of plasmid pSUS28 comprising the Δ pex11C::ble deletion cassette was transformed into *P. chrysogenum hdfA* GFP.SKL protoplasts. Phleo⁺ transformants were selected and analysed by colony PCR using primers attb4-Ppex11c.fw+attb3-Tpex11c.rev. These should amplify a fragment of 4.0 kb upon correct deletion of *pex11C* and a 3.5 kb fragment with *hdfA* GFP.SKL DNA. Finally, Southern blotting was used to confirm correct deletion. Multiple independent deletion strains were obtained and used for further study.

Construction of a P. chrysogenum pex16 deletion strain (Δ pex16 GFP.SKL)

A plasmid allowing deletion of *P. chrysogenum pex16* was constructed using Multisite Gateway technology as follows: First, the 5' and 3' flanking regions of *pex16* were amplified by PCR with

primer combinations 5'flank16 For + 5'flank16 Rev and 3'flank16 For + 3'flank16 Rev, respectively, using DS17690 genomic DNA as template. The resulting 1.7 kb (5') and 1.6 kb (3') fragments were then recombined into the Gateway vectors pDONRTM P4-P1R and pDONRTM P2R-P3, resulting in plasmids pPex16-5'flank and pPex16-3'flank, respectively. These plasmids were recombined with plasmids pENTR221-niaD_{F1}-amdS-niaD_{F2} and pDESTTM R4-R3, resulting in the deletion construct pDELPex16. This plasmid was linearized with *Aat*II and transformed into *P. chrysogenum hdfA* GFP.SKL protoplasts. AmdS⁺ transformants were selected and analysed by colony PCR using primers 5'forPex16 + 5'revPex16 (expected fragment: 4.3 kb) and 3'forPex16 + 3'revPex16 (expected fragment: 3.7 kb). Finally, Southern blotting was used to confirm correct deletion. Multiple independent deletion strains were obtained and used for further study.

Construction of a *P. chrysogenum pex3* deletion strain ($\Delta pex3$ GFP.SKL)

A plasmid allowing deletion of *P. chrysogenum pex3* was constructed using Multisite Gateway technology as follows: First, the 5' and 3' flanking regions of *pex3* were amplified by PCR with primer combinations LMOp100 + LMOp101 and LMOp102 + LMOp103, respectively, using DS17690 genomic DNA as template. The resulting 1.4 kb (5') and 1.5 kb (3') fragments were then recombined into the Gateway vectors pDONRTM P4-P1R and pDONRTM P2R-P3, resulting in plasmids pLMO 5' flank PEX3 and pLMO 3' flank PEX3, respectively. These plasmids were recombined with plasmids pENTR221-niaD_{F1}-amdS-niaD_{F2} and pDESTTM R4-R3, resulting in the deletion construct pLMO PEX3del. This plasmid was linearized with *Aat*II and transformed into *P. chrysogenum hdfA* GFP.SKL protoplasts. AmdS⁺ transformants were selected and analysed by colony PCR using primers LMOp109 + LMOp106 (expected fragment: 3.2 kb) and analysed by fluorescence microscopy. Multiple independent deletion strains were obtained and used for further study.

Construction of a *P. chrysogenum* strain deleted for both *pex11* and *pex11B* ($\Delta pex11 \Delta pex11B$ GFP.SKL)

To create a $\Delta pex11 \Delta pex11B$ double deletion mutant, we transformed protoplasts of one of the $\Delta pex11B$ GFP.SKL mutants with *Aat*II-linearized plasmid pBBK-017 carrying the $\Delta pex11::niaD_{F1}-amdS-niaD_{F2}$ cassette. AmdS⁺ transformants were selected, purified and

analysed by colony PCR using the primer combination BB-JK053+5-prime-niaD-return. This should only amplify a fragment of 1.35 kb upon correct deletion of *pex11*. Southern blotting was used to confirm correct deletion of *pex11*. Multiple independent double deletion strains were obtained and used for further study.

Construction of P. chrysogenum strains deleted for both pex11 and pex11C ($\Delta pex11 \Delta pex11C$ GFP.SKL and $\Delta pex11 \Delta pex11C$ GFP.SKL (AmdS⁻))

To create a $\Delta pex11 \Delta pex11C$ double deletion mutant, we transformed protoplasts of one of the $\Delta pex11C$ GFP.SKL mutants with *Aat*II-linearized plasmid pBBK-017 carrying the $\Delta pex11::niaD_{F1}-amdS-niaD_{F2}$ cassette. AmdS⁺ transformants were selected, purified and analysed by colony PCR using the primer combination BB-JK053+PgpdA.rev. This should only amplify a fragment of 2.9 kb upon correct deletion of *pex11*. Southern blotting was used to confirm correct deletion of both *pex11* and *pex11C*. Multiple independent double deletion strains were obtained and used for further study.

In the $\Delta pex11C \Delta pex11$ GFP.SKL double deletion strains, the *amdS* marker is flanked by a 1.5 kb repeat comprising part of *P. chrysogenum niaD* (F1 and F2), allowing loss of the marker by replication slippage. To obtain *amdS*-free strains, six independently isolated $\Delta pex11C \Delta pex11$ GFP.SKL strains were placed on sporulation agar and streaked out to single spore on fluoroacetamide (FlAc) plates. The resulting FlAc⁺ colonies were purified and re-tested on acetamide-containing plates. Southern blotting was used to confirm that in FlAc⁺/AmdS⁻ colonies the *amdS* gene had indeed been lost. Multiple independent $\Delta pex11 \Delta pex11C$ GFP.SKL (AmdS⁻) mutants were obtained.

Construction of a P. chrysogenum strain deleted for both pex11B and pex11C ($\Delta pex11B \Delta pex11C$ GFP.SKL)

To create a $\Delta pex11B \Delta pex11C$ GFP.SKL double deletion mutant, we transformed protoplasts of one of the $\Delta pex11C$ GFP.SKL mutants with *Apa*I-linearized plasmid pBBK-020 carrying the $\Delta pex11B::niaD_{F1}-amdS-niaD_{F2}$ cassette. AmdS⁺ transformants were selected, purified and analysed by colony PCR using the primer combination BB-JK025 met 5-prime-niaD-return. This should only amplify a fragment of 2.5 kb upon correct deletion of *pex11B*. Southern blotting was

used to confirm correct deletion of *pex11B*. Multiple independent double deletion strains were obtained and used for further study.

Construction of P. chrysogenum strains deleted for pex11, pex11B and pex11C ($\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL and $\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL (AmdS⁻))

To create a $\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL triple deletion mutant, protoplasts of one of the $\Delta pex11 \Delta pex11C$ GFP.SKL (AmdS⁻) double mutants were transformed with *ApaI*-linearized plasmid pBBK-020 carrying the $\Delta pex11B:: niaD_{F1}-amdS-niaD_{F2}$ cassette. AmdS⁺ transformants were selected, purified and analysed by colony PCR using the primer combinations BB-JK25 + 5-prime-niaD-return (2.6 kb) and BB-JK026 + 3-prime-niaD-forward (2.6 kb), which should only give a fragment of the indicated size upon correct recombination at the *pex11B* locus. Southern blotting was used to confirm correct deletion of *pex11*, *pex11B* and *pex11C*. Multiple independent triple deletion strains were obtained and used for further study.

To obtain *amdS*-free strains, one of the a $\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL triple deletion mutants was placed on sporulation agar and streaked out to single spore on FIAc plates. Colony PCR using primers LMOp071f and LMOp072r was used to confirm that the *amdS* gene had been lost (should amplify a fragment of 1.9 kb). Multiple independent $\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL (AmdS⁻) mutants were obtained.

Construction of a P. chrysogenum strain deleted for pex11, pex11B, pex11C and pex16 ($\Delta pex11 \Delta pex11B \Delta pex11C \Delta pex16$ GFP.SKL)

To obtain the quadruple mutant $\Delta pex11 \Delta pex11B \Delta pex11C \Delta pex16$ GFP.SKL, protoplasts of *P. chrysogenum* $\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL (AmdS⁻) were transformed with *AatII* linearized pDELPex16. AmdS⁺ transformants were selected and correct deletion was demonstrated by colony PCR using primers 5'forPex16 + 5'revPex16 (expected fragment: 4.3 kb) and 3'forPex16 + 3'revPex16 (expected fragment: 3.7 kb). Southern blotting was used to confirm correct deletion of *pex16*. Multiple independent $\Delta pex11 \Delta pex11B \Delta pex11C \Delta pex16$ GFP.SKL strains were obtained and used for further study.

Construction of a *P. chrysogenum* strain deleted for *pex11*, *pex11B*, *pex11C* and *vps1* ($\Delta pex11 \Delta pex11B \Delta pex11C \Delta vps1$ GFP.SKL)

For the construction of a $\Delta vps1$ deletion cassette, the 5' and 3' flanking regions of the *vps1* gene were amplified by PCR using primers KAR001+KAR002 and KAR003+KAR004, respectively, using *P. chrysogenum* WT genomic DNA as template. The obtained 2.05 kb (5') and 1.95 kb (3') fragments were then recombined into the Gateway vectors pDONRTM P4-P1R and pDONRTM P2R-P3, resulting in plasmids pKAR-002 and pKAR-003, respectively. These plasmids were recombined with plasmids pENTR221-*niaD*_{F1}-*amdS*-*niaD*_{F2} and pDESTTM R4-R3, resulting in the deletion construct pBBK-023.

To create a *P. chrysogenum* strain deleted for *pex11*, *pex11B*, *pex11C* and *vps1*, plasmid pBBK-023 was linearized with *Aat*II and transformed into protoplasts of *P. chrysogenum* $\Delta pex11 \Delta pex11B \Delta pex11C$ GFP.SKL (*AmdS*⁻). *AmdS*⁺ transformants were selected and correct integration of the deletion cassette was determined by colony PCR using primers LMOp105 + LMOp106 (expected fragment: 3.7 kb) and LMOp107 + LMOp108 (expected fragment: 3.7 kb). Multiple independent deletion strains were obtained and used for further study.

2. *P. chrysogenum* strains overexpressing *pex11*, *pex11B*, *pex11C* or *pex16*

Control strain *P. chrysogenum* DS17690 expressing GFP.SKL (DS17690 GFP.SKL)

A derivative of the wild type *P. chrysogenum* strain DS17690 constitutively expressing *GFP.SKL* was constructed as follows: A 2.2 kb *Not*I fragment from plasmid pWHM-001, comprising the *P*_{*gpdA*}-*GFP.SKL*-*T*_{*penDE*} expression cassette, and a 6.2 kb *Not*I-*Spe*I fragment from pNiGANi, containing a *PgpdA-amdS* expression cassette flanked by *niaD* repeats, were co-transformed into *P. chrysogenum* DS17690 protoplasts. Multiple green fluorescent *AmdS*⁺ co-transformants were selected for further use.

Construction of a strain overexpressing *pex11* (GFP.SKL *pex11*+++)

For overexpression of *pex11* in *P. chrysogenum* DS17690, a 2.3 kb *Not*I fragment comprising the *P*_{*pcbC-pex11*}-*T*_{*penDE*} overexpression cassette was isolated from plasmid pGBRH2-PEX11. The fragment was co-transformed together with a 2.2 kb *Not*I fragment from plasmid pWHM-001, comprising the *P*_{*gpdA*}-*GFP.SKL*-*T*_{*penDE*} expression cassette, and a 6.2 kb *Not*I-*Spe*I fragment from pNiGANi, containing the *PgpdA-amdS* expression cassette flanked by *niaD* repeats, into

DS17690 protoplasts. Green fluorescent AmdS⁺ co-transformants were selected and analysed by colony PCR using the *pex11* cassette-specific primers BB-JK007 and BB-JK008 for the presence of the overexpression cassette. These primers will amplify a 962 bp DNA fragment from the genomic clone (with two introns) and a 688 bp fragment when the *pex11* overexpression cassette (cDNA) is present. Overproduction of Pex11 in cDNA positive transformants was checked by western blotting using specific antibodies against Pex11. Multiple green fluorescent strains producing strongly enhanced levels of Pex11 were identified and used for further study.

Construction of a strain overexpressing pex11B (GFP.SKL pex11B+++)

A *P_{pcbC}-pex11B-T_{penDE}* overexpression cassette was prepared as follows: The *pex11B* coding sequence with stop codon was amplified by PCR with oligonucleotides BB-JK012 and BB-JK014 using DNA of a *P. chrysogenum* cDNA library as template [5]. The obtained 736 bp PCR product was recombined into pDONRTM 221, resulting in pBBK-009. Subsequently, pENTR41-PpcbC, pBBK-009, pENTR23-His8.TpenDE and pDESTTM R4-R3/AMDS were recombined resulting in plasmid pROK-014. This plasmid was linearized with *AatII* and co-transformed with a 2.2 kb *NotI* fragment from pWHM-001, comprising the *P_{gpdA}-GFP.SKL-T_{penDE}* expression cassette into *P. chrysogenum* DS17690 protoplasts. Green fluorescent AmdS⁺ co-transformants were selected and analysed by colony PCR using the *pex11B* cassette-specific primers BB-PEX11B-Fw and BB-PEX11B-Rv for the presence of the overexpression cassette. These primers will amplify a 804 bp DNA fragment from the genomic clone (with two introns) and a 674 bp fragment when the overexpression cassette (cDNA) is present. Overproduction of Pex11B in cDNA positive transformants was checked by western blotting using specific antibodies against Pex11B. Multiple green fluorescent strains producing high levels of Pex11B were identified and used for further study.

Construction of a strain overexpressing pex11C (GFP.SKL pex11C+++)

A *P_{pcbC}-pex11C-T_{penDE}* overexpression cassette was prepared as follows: The *pex11C* coding sequence with its stop codon was amplified by PCR with oligonucleotides BB-JK029 and PEX11Cst.rev using plasmid pBBK-012 (*see below*) DNA as template. The obtained 976 bp PCR product was recombined into pDONRTM 221, resulting in pROK-013. Subsequently, plasmids pENTR41-PpcbC, pROK-013, pENTR23-His8.TpenDE and pDESTTM R4-R3/AMDS

were recombined resulting in plasmid pROK-015. This plasmid was linearized with *AatII* and co-transformed with a 2.2 kb *NotI* fragment from pWHM-001, comprising the $P_{\text{gpdA}}\text{-GFP.SKL-T}_{\text{penDE}}$ expression cassette into *P. chrysogenum* DS17690 protoplasts. Green fluorescent AmdS⁺ co-transformants were selected and analysed by colony PCR using the *pex11C* cassette-specific primers BB-PEX11C-Fw and BB-PEX11C-Rv for the presence of the overexpression cassette. These primers will amplify a 739 bp DNA fragment from the genomic clone (with one intron) and a 675 bp fragment when the overexpression cassette (cDNA) is present. Overproduction of Pex11C in positive transformants was checked by western blotting using specific antibodies against Pex11C. Multiple green fluorescent strains producing high levels of Pex11C were identified and used for further study.

3. Construction of strains allowing localization of Pex3, Pex11, Pex11B, Pex11C and Pex16

Construction of a strain producing Pex11-mGFP (DsRed.SKL *pex11.mGFP*)

A plasmid producing *P. chrysogenum* Pex11 fused C-terminally with monomeric green fluorescent protein (mGFP) was constructed using Multisite Gateway technology.

First, we constructed a general Gateway vector comprising the *mGFP* sequence and the *penDE* terminator region as follows: By PCR with the oligonucleotides BamHI-mGFP-Fw and blunt-mGFP-Rv we amplified the *mGFP* gene using plasmid pCGCN-FAA4 as template. The 732 bp PCR fragment was digested with *BamHI* and inserted between the *BamHI* and *SmaI* sites of plasmid pGBRH2, resulting in plasmid pGBRH2-mGFP. Subsequently, we amplified the *mGFP-T}_{\text{penDE}}* region from this plasmid with oligonucleotides XFP-B2f and AT-B3R and recombined the fragment into pDONRTM P2R-P3, resulting in plasmid pENTR23-mGFP-TpenDE.

The *pex11* coding sequence lacking the stop codon was amplified by PCR using oligonucleotides BB-JK001 and BB-JK002 and DNA from plasmid pUC19-PcPEX11 as template. The obtained 778 bp PCR product was recombined into the entry vector pDONRTM 221, resulting in pBBK-002. Subsequently, vectors pBBK-005, pBBK-002, pENTR23-mGFP-TpenDE and pDESTTM R4-R3 were recombined resulting in plasmid pROK-007. A 3.4 kb *NcoI-PvuI* fragment comprising the $P_{\text{pex11}}\text{-pex11.mGFP-T}_{\text{penDE}}$ expression cassette from pROK-007 was co-transformed with a 6.2 kb *NotI-SpeI* fragment from plasmid pNiGANi containing a $P_{\text{gpdA}}\text{-amdS}$ expression cassette flanked by *niaD* repeats into protoplasts of *P. chrysogenum* DsRed.SKL (AmdS⁻), in which peroxisomes are marked by the red fluorescent DsRed.SKL protein. AmdS⁺

transformants were isolated and analysed using fluorescence microscopy. Multiple green + red fluorescent transformants were selected for further study.

Construction of strains producing Pex11B-mGFP (DsRed.SKL pex11B.mGFP and sec63.mCherry pex11B.mGFP)

A plasmid producing *P. chrysogenum* Pex11B fused C-terminally with mGFP was constructed using Multisite Gateway technology. The *pex11B* coding sequence lacking the stop codon was amplified by PCR using oligonucleotides BB-JK012 and BB-JK013 and DNA from a *P.*

chrysogenum cDNA library [5] as template. The obtained 733 bp PCR product was recombined into the entry vector pDONRTM 221, resulting in pBBK-008. Subsequently, vectors pBBK-005, pBBK-008, pENTR23-mGFP-T_{penDE} were recombined with pDESTTM R4-R3/AMDS(NotI) resulting in plasmid pBBK-019, which contains a *P_{pex11}-pex11B.mGFP-T_{penDE}* expression cassette. The plasmid was linearized with *NotI* and transformed into protoplasts of *P.*

chrysogenum DsRed.SKL (AmdS⁻), in which peroxisomes are marked by the red fluorescent DsRed.SKL protein. AmdS⁺ transformants were isolated and analysed using fluorescence microscopy. Multiple green + red fluorescent transformants were selected for further study.

To demonstrate co-localization of *P. chrysogenum* Pex11B with the endoplasmic reticulum (ER), we constructed a plasmid expressing a *sec63.mCherry* fusion gene as follows: First, we identified the *P. chrysogenum* Sec63 ortholog by BlastP analysis at the National Center for Biotechnology Information (NCBI) using the *S. cerevisiae* Sec63 protein sequence as a query, which identified the protein Pc16g14390. Subsequently, we amplified the region comprising the putative *P. chrysogenum* *sec63* (*Pc16g14390*) promoter and coding sequence lacking a stop codon by PCR with oligonucleotides Psec63-Fw + sec63-no stop-Rv using *P. chrysogenum* DS17690 genomic DNA as template. The 3.5 kb PCR fragment was digested with *Asp718i* and *BamHI* and cloned between the *Asp718i* and *BamHI* sites of plasmid pGBRH2-eGFP, resulting in plasmid pBBK-021, which carries a *P_{sec63}-sec63.GFP-T_{penDE}* expression cassette.

Subsequently, we isolated the *mCherry* gene as a 740 bp *BglII-SmaI* fragment from plasmid pRSA01 and cloned it between the *BamHI* and *SmaI* sites of pBBK-021, resulting in plasmid pBBK-022. This plasmid carries a *P_{sec63}-sec63.mCherry-T_{penDE}* expression cassette. To create a strain expressing both *sec63.mCherry* and *pex11B.mGFP*, plasmids pBBK-022 and pBBK-019 were both linearised with *NotI* and co-transformed into *P. chrysogenum* DS17690 protoplasts.

AmdS⁺ transformants were isolated and analysed using fluorescence microscopy. Multiple green + red fluorescent transformants were selected for further study.

Construction of a strain producing a Pex11C.mGFP fusion protein (DsRed.SKL pex11C.mGFP)

A plasmid containing *P. chrysogenum* Pex11C fused C-terminally with mGFP was constructed using Multisite Gateway technology. The *pex11C* coding sequence lacking the stop codon was amplified by PCR using oligonucleotides BB-JK029 and BB-JK030 and DNA from a *P. chrysogenum* cDNA library [5] as template. The obtained 973 bp PCR product was recombined into the entry vector pDONRTM 221, resulting in pBBK-012. Subsequently, vectors pBBK-005, pBBK-012, pENTR23-mGFP-TpenDE and pDESTTM R4-R3 were recombined resulting in plasmid pBBK-016, which contains a P_{pex11}-*pex11C.mGFP-T_{penDE}* expression cassette. pBBK-016 was linearized with *EcoRV* and co-transformed with a 6.2 kb *NotI-SpeI* fragment from plasmid pNiGANi containing a P_{gpdA}-*amdS* expression cassette flanked by *niaD* repeats into *P. chrysogenum* DsRed.SKL (AmdS⁻) protoplasts, in which peroxisomes are marked by the red fluorescent DsRed.SKL protein. AmdS⁺ transformants were isolated and analysed using fluorescence microscopy. Multiple green + red fluorescent transformants were selected for further study.

Construction of strains producing a Pex16.mGFP fusion protein (DsRed.SKL pex16.mGFP and sec63.mCherry pex16.mGFP)

A plasmid containing *P. chrysogenum* Pex16p fused C-terminally with mGFP was constructed using Multisite Gateway technology. The Pex16 coding sequence lacking the stop codon was amplified by PCR with oligonucleotides Pex16CDSfor and Pex16CDSrev using DNA from a *P. chrysogenum* cDNA library [5] as template. The obtained 1.2 kb PCR product was recombined into the entry vector pDONRTM 221, resulting in pDONR221-Pex16. Subsequently, vectors pENTR41-PgdpA, pDONR221-Pex16, pENTR23-mGFP-TpenDE and pDESTTM R4-R3/AMDS were recombined resulting in plasmid pPex16-mGFP, which contains a P_{gpdA}-*pex16.mGFP-T_{penDE}* expression cassette. Plasmid pPex16-mGFP was linearized with *AatII* and transformed into *P. chrysogenum* DsRed.SKL (AmdS⁻) protoplasts, in which peroxisomes are marked by the red fluorescent DsRed.SKL protein. AmdS⁺ transformants were isolated and analysed using

fluorescence microscopy. Multiple green + red fluorescent transformants were selected for further study.

To determine possible co-localization of *P. chrysogenum* Pex16 with the ER, we constructed a *P. chrysogenum* strain producing the ER marker Sec63.mCherry and Pex16.mGFP by co-transformation of *P. chrysogenum* DS17690 with *Aat*II linearized pPex16-mGFP and *Not*I linearized pBBK-022. AmdS⁺ transformants were isolated and analysed using fluorescence microscopy. Multiple green + red fluorescent transformants were selected for further study.

References:

1. Hillenga DJ, Versantvoort HJ, Driessen AJ, Konings WN. 1994. Structural and functional properties of plasma membranes from the filamentous fungus *Penicillium chrysogenum*. Eur J Biochem 224, 581-587.
2. Cantoral JM, Diez B, Barredo JL, Alvarez E, Martín JF. 1987. High-frequency transformation of *Penicillium chrysogenum*. Biotechnology 5, 494-497.
3. Royer JC, Christianson LM, Yoder WT, Gambetta GA, Klotz AV, Morris CL, Brody H, Otani S. 1999. Deletion of the trichodiene synthase gene of *Fusarium venenatum*: two systems for repeated gene deletions. Fungal Genet Biol 28, 68-78.
4. Van Dijken JP, Otto R, Harder W. 1976. Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. Arch Microbiol 111, 137-144.
5. Kiel JA, Hilbrands RE, Bovenberg RA, Veenhuis M. 2000. Isolation of *Penicillium chrysogenum* *PEX1* and *PEX6* encoding AAA proteins involved in peroxisome biogenesis. Appl Microbiol Biotechnol 54, 238-242.